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Fibronectin Synthesis is a Marker for Peritubular Cell Contaminants in Sertoli Cell-Enriched Cultures

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ABSTRACT

With indirect immunofluorescent microscopic techniques, we have shown that fibronectin is distributed primarily in or along the basal lamina of the seminiferous tubule boundary tissue in sections of testes from 20-day-old rats. Purified rat Sertoli cell-enriched aggregates, maintained in culture in the presence or absence of serum, exhibit no detectable immunofluorescence with fibronectin antibody, whereas purified peritubular cells in culture do have a positive reaction to fibronectin antibody. Peritubular cells in culture incorporate [^{35}S]methionine into fibronectin which can be immunoprecipitated with a fibronectin antiserum, but Sertoli cells do not. We have used various criteria to estimate the degree of purity of Sertoli cell-enriched preparations. The presence of peritubular myoid cells in conventional Sertoli cell-enriched aggregates, cultured in the presence or absence of serum, can be detected with transmission electron microscopic examination, by the Feulgen staining procedure, and by the immunocytochemical identification of fibronectin. We describe a technique to purify Sertoli cells in conventional Sertoli cell-enriched preparations by treatment with hyaluronidase, resulting in a lesser number of peritubular cells by the above criteria, even in preparations cultured in the presence of serum. Data presented suggest that some of the products previously attributed exclusively to Sertoli cells in Sertoli cell-enriched preparations, particularly those cultured in the presence of serum, may have been contributed by peritubular cells.

INTRODUCTION

Primary cultures of Sertoli cells have proven useful in a variety of investigations, especially those designed to determine the influences of follicle-stimulating hormone (FSH) and androgens on the control of formation of various products by Sertoli cells (for review, see Fritz, 1978). After subjecting fragments of testes from immature rats to treatment with trypsin and collagenase, aggregates prepared contain approximately 70% Sertoli cells (Dorrington et al., 1975; Tung et al., 1975; Fritz et al., 1975). After these heterogeneous but enriched Sertoli cell aggregates have been dispersed and maintained in culture on plastic surfaces in a chemically defined medium for 3 or more days, the percentage of Sertoli cells increases to approximately 90%, primarily because most of the residual germ cells do not remain attached, and other cell types in testis are apparently less well maintained in a serum-free medium (Tung and Fritz, 1977).

Sertoli cells in primary culture, maintained in a relatively simple chemically defined medium, continue to respond to FSH for at least 2 weeks with increased rates of production of androgen-binding protein (ABP) (Fritz et al., 1976); plasminogen activator (Lacroix and Fritz, 1977; 1982); and transferrin (Skinner and Griswold, 1982). After 2 to 3 weeks in culture in the absence of serum or a peritubular cell "feeder layer," rates of formation of these products diminish in the Sertoli cell preparations. Even when serum is added to the medium, primary cultures of Sertoli cells begin to deteriorate within a month after being plated on plastic dishes, and they detach from the substratum. Peritubular myoid cells and fibroblasts, present as contaminants in the Sertoli cell-enriched aggregates (Fritz et al., 1975; Tung and Fritz, 1977), will proliferate if serum is present and will become the predominant cell types unless inhibitors of DNA synthesis such as cytosine arabinoside are added (Tung et al., 1980).

When Sertoli cell aggregates are plated on top of a layer of peritubular myoid cells, the

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properties of each cell type are altered considerably. In addition to greater production of ABP for a more sustained period by Sertoli cells in coculture with peritubular cells (Tung and Fritz, 1980; Hutson and Stocco, 1981), both cell types survive longer. The two cell types interact to form basement membranes and a unique architectual arrangement which resembles the histotype of seminiferous tubules (Tung and Fritz, 1980). These effects of large numbers of peritubular cells on Sertoli cells in culture raise questions concerning the potential influence of lesser numbers of peritubular cells known to be present in conventional Sertoli cell-enriched preparations (Fritz et al., 1975; Tung and Fritz, 1977). Is it possible that contaminating peritubular cells could contribute to the formation of products previously attributed exclusively to Sertoli cells in culture? Does the presence of peritubular myoid cells alter the responses of Sertoli cells to hormones?

Motivated by these considerations, we have investigated methods by which the number of peritubular cells in Sertoli cell preparations can be reduced or eliminated. Observations to be reported demonstrate that hyaluronidase treatment of the conventional preparations considerably lessens contamination of the Sertoli cell aggregates by peritubular cells, and that even when cultured in the presence of serum, the more purified preparations have only small numbers of peritubular cells. Data to be presented demonstrate that fibronectin, a component of extracellular matrix (Hynes and Bye, 1974; Ruoslahti and Vaheri, 1974; Yamada and Weston, 1974) is produced by peritubular cells but not by Sertoli cells. Fibronectin synthesis, as assessed immunocytochemically or by examination of radiolabeled proteins, is shown to provide a useful marker for the presence of peritubular cells in Sertoli cell-enriched preparations cultured in the presence or absence of serum. We have correlated this with an estimation by microscopic analysis of the number of peritubular cells present in conventional and purified Sertoli cell-enriched preparations cultured in the presence or absence of serum.

MATERIALS AND METHODS

Preparation and Culture of Sertoli Cell-Enriched Aggregates and Peritubular Myoid Cells

Conventional cultures of Sertoli cell-enriched preparations were isolated from testes of 20-day-old Wistar rats according to the procedures of Dorrington and Fritz (1975), with minor changes (Tung et al., 1975; Tung and Fritz, 1977). To purify these preparations, we modified the procedures as follows. After trypsinization of decapsulated testes in the presence of DNase as previously described (Dorrington et al., 1975), we subjected the seminiferous tubule segments to treatment with a mixture of collagenase (1 mg/ml, Type I, Sigma Chemical Co., St. Louis, MO) and bovine testicular hyaluronidase (1 mg/ml, Type 1-S, Sigma) in Hanks' buffer for 30 min with gentle agitation at 32°C. The tubule fragments, extensively washed with Hanks' buffer, were harvested by low-speed centrifugation, and then digested with hyaluronidase (1 mg/ml) alone for another 30 min. The resulting aggregates were washed twice with Hanks' buffer containing 1% bovine serum albumin (BSA; Sigma), and were dispersed by rigorous agitation with a Pasteur pipette in Ca^{2+}-and Mg^{2+}-free Hanks' buffer containing 0.1 mM ethylene glycol bis (β-aminoethyl ether)-N,N,N′,N′-tetraacetate (EGTA; Sigma). Cell aggregates were washed twice more with 1% BSA, and then plated at desired densities onto polystyrene tissue culture Petri dishes. For sparse cultures, ordinary Sertoli cell-enriched or purified preparations were seeded at 200 aggregates/100 mm^2, with each aggregate containing about 100 cells. For confluent cultures, they were plated at 1500 aggregates/100 mm^2. The medium and culture conditions employed were identical to those previously described (Tung et al., 1975). Peritubular cells were prepared from testes of 20-day-old Wistar rats, according to procedures described by Tung and Fritz (1977), and were cultured in the presence or absence of 10% calf serum, in Eagle's modified essential minimal medium (MEM), as specified in the legends to the figures.

In other experiments, Sertoli cell preparations were cultured for 24 h in serum-free MEM made deficient in methionine, containing [^{35}S]methionine (10 μCi/ml, New England Nuclear Corp., Montreal, Quebec). Radiolabeled proteins in the medium were collected for analysis by gel electrophoresis before and after immunoprecipitation with fibronectin antiserum by procedures described below. Peritubular cell-radiolabeled proteins were obtained in a similar manner from the medium in which subcultured confluent peritubular cells had been incubated for 24 h in the presence of [^{35}S]methionine. Prior to addition of the [^{35}S]methionine, peritubular cells had been cultured for a minimum of 48 h in serum-free MEM.

Indirect Immunofluorescent Techniques

We used a mouse monoclonal antibody (IgG), directed against porcine plasma fibronectin, generously provided by Dr. J. Aubin (MRC Group in Periodontal Physiology, University of Toronto). The monoclonal antibody producing hybridoma was cultured in serum-free medium, and the IgG in the culture medium was collected and used without dilution. Bulk absorption was carried out with rat plasma fibronectin (Calbiochem, La Jolla, CA). Undiluted antibody was absorbed for 60 min at 4°C (2 vol of antibody per vol of absorbing material). Absorption was repeated until activity (determined by the indirect immunofluorescent technique) of the antibody was abolished. The samples were centrifuged at 10,000 × g for 30 min at 4°C, after which the clear supernatant fractions were dispersed in small aliquots and stored at −30°C.

Sertoli cell-enriched preparations grown on glass coverslips were fixed after various periods of culture with 3% paraformaldehyde in Ca^{2+} and Mg^{2+}-free Hanks' buffer for 20 min at room temperature, followed by immersion in acetone at −10°C for 7 min. Preparations were then washed with multiple changes of phosphate-buffered saline (PBS) at 4°C. The specimens were incubated with the antibody solution in serial dilutions in PBS, and again washed with PBS containing 0.05% Tween 20 (Sigma). Affinity purified $F(AB')_2$ fragments of goat immunoglobulins conjugated with fluorescein isothiocyanate (FITC) (Cappel Lab., West Chester, PA), diluted 1:30 with PBS, was added to the washed specimens for another 30 min, followed by additional washes with PBS containing 0.05% Tween 20. The preparations were mounted and examined with fluorescent microscopy as previously described (Tung and Fritz, 1978). In other experiments, 10-μm frozen sections of seminiferous tubules were prepared with an Ames cryostat, and used either for immunofluorescent or light microscopic examination after being counterstained with Paragon multiple stain (Paragon C and C Co., Bronx, NY).

Morphological Techniques

Cell cultures were examined periodically using a Nikon inverted microscope with phase optics, and representative dishes or fields were photographed. For transmission electron microscopy (TEM), the samples were fixed in 3% glutaraldehyde in 0.1 M Millonig phosphate buffer and processed as in our previous investigations (Tung et al., 1975). For scanning electron microscopy (SEM), samples were fixed in 2% glutaraldehyde in the same buffer and processed for observations (Tung et al., 1976).

Gel Electrophoresis and Immunoprecipitation Procedures

Fibronectin immunoprecipitation was done with a double-antibody precipitation procedure previously described (Skinner and Griswold, 1983a). To 200 μl of culture medium containing radiolabeled proteins we added either 25 μl of goat antiserum against rat fibronectin (Calbiochem) or 100 μl of monoclonal mouse anti-pig plasma fibronectin (1 mg/ml), and 200 μl of an immunoprecipitation buffer (50 mM Tris, pH 7.5, 0.15 M NaCl, 1% Triton X-100, and 6 μM phenyl methyl sulfonylfluoride). After the mixture had been incubated for 24 h at 4°C, 20 μl of a rabbit anti-goat IgG or rabbit anti-mouse igG, respectively, were added, and the mixture was incubated for an additional 24 h at 4°C. The precipitate was then centrifuged at 13,000 × *g* for 15 min at 4°C. The pellet was washed three times with immunoprecipitation buffer and then dissolved in electrophoresis sample buffer, consisting of 30% glycerol, 3% sodium dodecyl sulfate (SDS), 3% β-mercaptoethanol, 0.01% bromophenyl blue, and 0.1 M Tris, pH 6.8. Electrophoretic analysis was performed with 5 to 15% polyacrylamide gradient slab gels containing SDS, using the Laemmli (1970) buffer system. Fluorographic analysis of the polyacrylamide gel was performed by a procedure using diphenyloxazole in acetic acid (Skinner and Griswold, 1983b).

RESULTS

Presence of Fibronectin in Peritubular Boundary Tissue

Indirect immunofluorescent microscopy was employed to localize fibronectin in cryostat sections of testes from 20-day-old rats. Using monoclonal antibody against fibronectin, we observed that fibronectin was distributed primarily in or along the basal lamina of the seminiferous tubule boundary tissue and in interstitial tissues (Fig. 1A and C). No detectable fibronectin was evident within the luminal compartment of the seminiferous tubule. Monoclonal fibronectin antibody preadsorbed with fibronectin did not react (Fig. 1B and D). To determine if fibronectin present in the boundary tissue could have been formed by cells on either side of the basal lamina, we next examined cultures of testicular cells for the presence of fibronectin.

Deposition of Fibronectin by Testicular Peritubular Cells but Not by Sertoli Cells in Culture

Newly isolated peritubular cells did not initially react with monoclonal fibronectin antibody (data not shown). However, peritubular cells, maintained for 1 or more days in culture, began to exhibit a positive reaction to fibronectin antibody (Fig. 2B and E). In contrast, cultures of purified Sertoli cell-enriched preparations, which had previously been treated with hyaluronidase, were consistently negative (Fig. 2A and D). Monoclonal fibronectin antibody preadsorbed with rat plasma fibronectin did not react with peritubular cells (Fig. 2C and F), confirming the cross-reactivity between porcine fibronectin antibody and fibronectin produced by rat peritubular cells.

In peritubular cells cultured in the presence of serum, fibronectin deposition in the extracellular space is clearly evident in a fibrillar-like arrangement (Fig. 3A). In peritubular cells cultured in the absence of serum, most of the fibronectin antibody-reactive material is located intracellularly in the perinuclear region (Fig. 3B and C).

Although fibronectin could not be detected in confluent cultures of purified Sertoli cell-enriched preparations subjected to hyaluronidase treatment (Figs. 2A,D and 4A,C), it was evident in confluent cultures of conventional Sertoli cell-enriched preparations not previously digested with hyaluronidase (Fig. 4B and D).

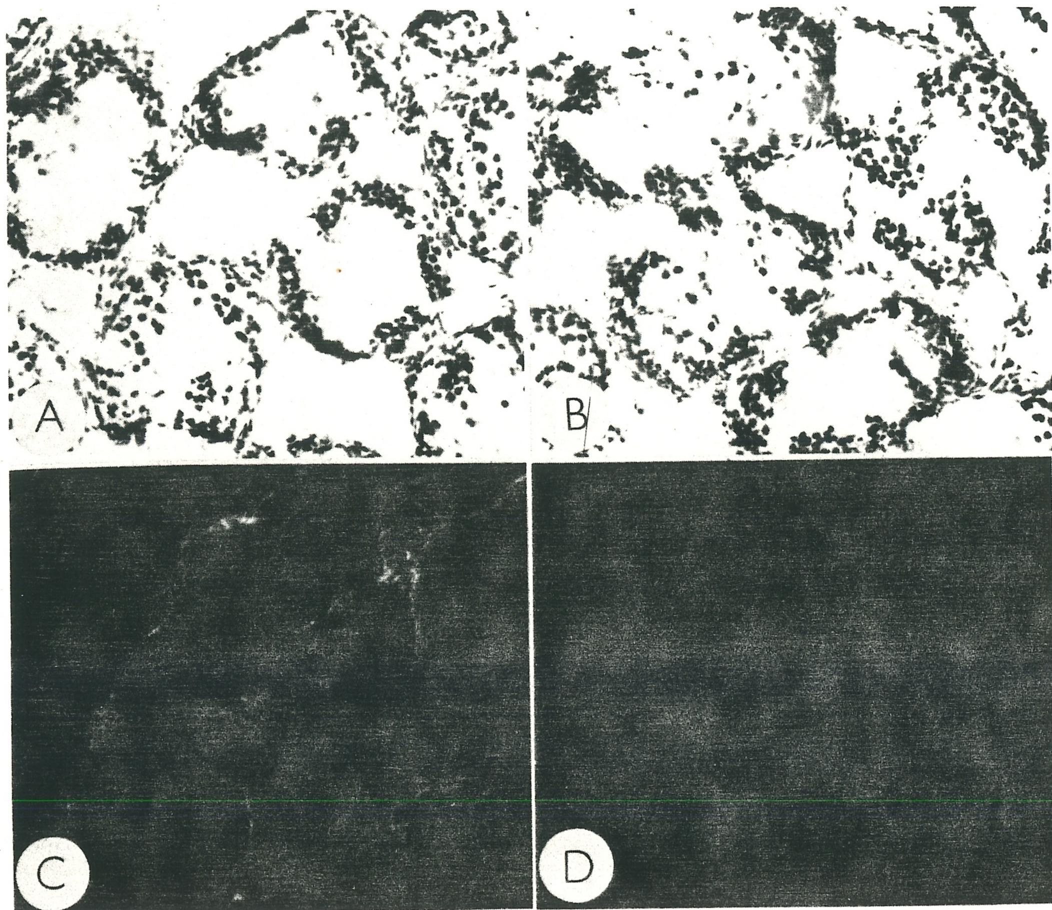


FIG. 1. Immunofluorescent micrographs (*C* and *D*) and phase-contrast microgarphs (*A* and *B*) of cryostat sections of testis from a 20-day-old rat. *A* and *C* show sections reacted with the monoclonal fibronectin antibody preparation. *B* and *D* show sections reacted with the same antiserum which had previously been adsorbed with rat plasma fibronectin. ×300.

Phase-contrast microscopic examination did not reveal the appearance of fibroblast-like cells (Fig. 4B). To examine the possibility that fibronectin could have been produced in these cultures by mesenchymal cells beneath the monolayer of Sertoli cells, we examined TEM sections. We observed the presence of attenuated myoid cells underlying Sertoli cells in nearly all conventional preparations of Sertoli cell-enriched aggregates not subjected to hyaluronidase treatment and cultured in the presence of serum (Fig. 5). The ultrastructure of these cells is similar to that described for peritubular myoid cells (Bressler and Ross, 1972, 1973; Tung and Fritz, 1975).

Incorporation of Labeled Amino Acids into Immunoprecipitable Fibronectin by Peritubular Cells but Not by Sertoli Cells in Culture

Purified Sertoli cells or subcultured peritubular cell preparations were incubated with [^{35}S] methionine, and labeled proteins released into the medium were analyzed (Fig. 6). The polyacrylamide gel electrophoretic profiles of proteins secreted by the two cell types were clearly different (Fig. 6A and E). Comparable profiles, varying only in intensity, were obtained when cells were cultured for various periods up to 7 days in the presence or absence of dbcAMP (0.1 mM); FSH (NIH S-15, 200 ng/ml); insulin

(5 μg/ml); retinol (0.35 μM); and testosterone (0.5 μM) (unpublished observations). Kissinger et al. (1982) have reported similar findings. Immunoprecipitation of the radiolabeled proteins in the culture medium with fibronectin antibody resulted in the detection of a protein of approximately 220,000 M_r (Fig. 6, lanes *B* and *C*), whereas nonimmune sera did not precipitate detectable amounts of material (Fig. 6, lane *D*). In contrast, addition of fibronectin antibody or nonimmune serum did not precipitate any material from Sertoli cell culture medium (Fig. 6, lanes *F* and *G*), confirming the absence of fibronectin synthesis by Sertoli cells. Protein released by Sertoli cells and by peritubular cells were subjected to gel electrophoresis and then stained with Coomassie blue. A major peritubular cell-secreted product comigrates with rat fibronectin, whereas no such protein is present in Sertoli cell medium (Fig. 6, lanes *H*, *I* and *J*).

Identification of Sertoli Cells and Peritubular Cells by Microscopic Examination of Feulgen-Stained Preparations in Culture

The presence of peritubular cells in Sertoli cell-enriched preparations may be assessed by TEM examination (Tung and Fritz, 1975), but

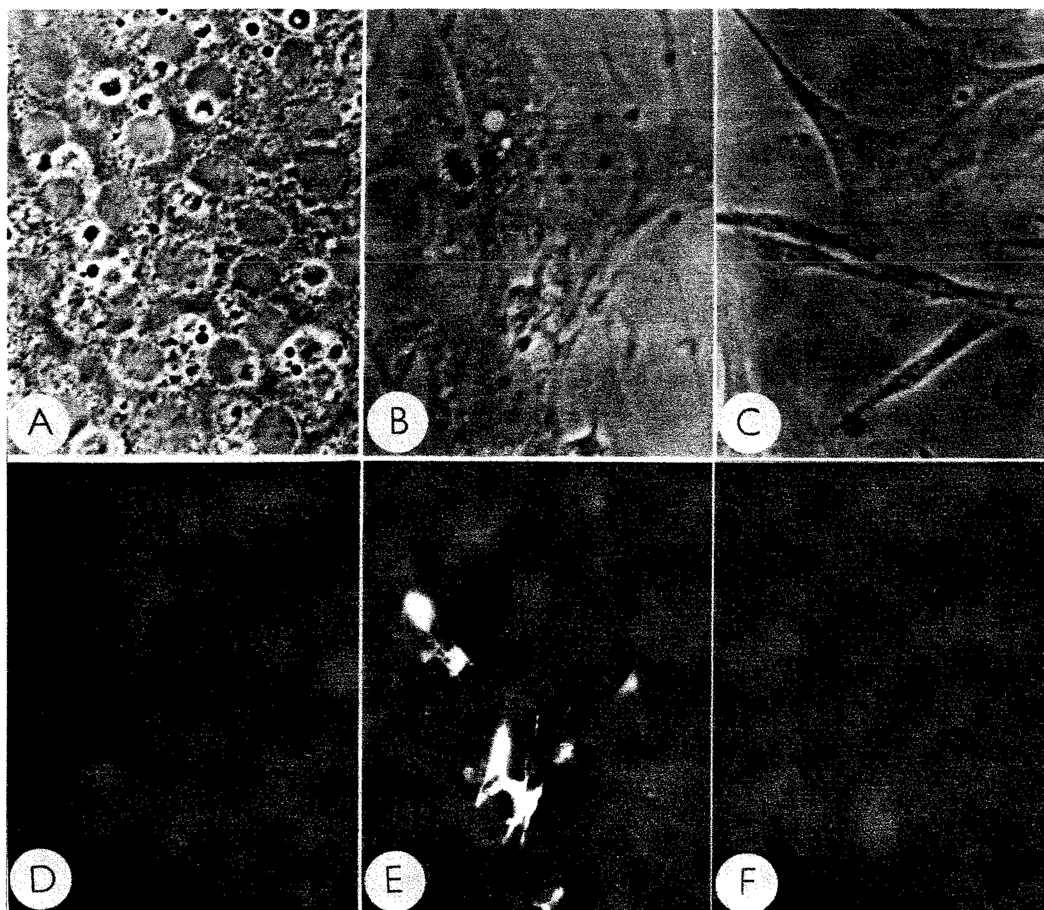


FIG. 2. Phase-contrast micrographs (*A*, *B*, *C*) and immunofluorescent micrographs (*D*, *E*, *F*) of cultures of purified Sertoli cell (*A*, *D*), and peritubular cell (*B*, *C*, *E*, *F*) preparations. All cells had been cultured in modified MEM containing 10% calf serum for 4 days. They were then fixed and reacted with monoclonal fibronectin antibody (*A*, *D*, *B*, *E*), or with the same antibody previously adsorbed with rat plasma fibronectin (*C*, *F*). Note Sertoli cells are negative (*A*, *D*), while peritubular cells (*B*, *E*) are positive to unadsorbed fibronectin antibody. Peritubular cells reacted with the same antiserum which had previously been adsorbed were negative (*C*, *F*). ×1200.

myoid cells are more difficult to discern by ordinary phase-contrast microscopic examination (compare Fig. 4B with Fig. 5). With the Feulgen staining procedure, however, it is possible to distinguish Sertoli cells from peritubular cells with relative ease (Fig. 7). The shapes of the nuclei of Sertoli cells are distinctive, frequently with perinucleolar bodies (Fig. 7B). In contrast, multiple small nucleoli are evident in peritubular myoid cells stained in the Feulgen reaction (Fig. 7A).

Using this procedure, we have observed that conventional Sertoli cell-enriched preparations cultured in serum which have been shown to contain peritubular cells by other criteria (Figs. 4B,D and 5), have peritubular cells readily recognizable by Feulgen stain microscopy (Fig. 7C and E). In contrast, purified Sertoli cell-enriched preparations contain only small numbers of peritubular cells (Figs. 4A and 7B,D). In other studies, we have observed that primary cultures of peritubular cells contain occasional Sertoli cells detectable in Feulgen stain preparations, and this is correlated with the absence of immunocytochemically detectable fibronectin in these cells (data not shown). Subcultured peritubular cells contain no Sertoli cells detectable by the Feulgen staining procedure (Fig. 7A). This absence of microscopically detectable Sertoli cells is correlated with the uniform presence of immunocytochemically detectable fibronectin (Fig. 2E).

The number of peritubular cells was determined in conventional and purified Sertoli cell-enriched preparations cultured for 6 days in the presence and absence of serum (Table 1). In the absence of serum, the number of peritubular cells was small in both conventional aggregates and in Sertoli cell aggregates subjected to hyaluronidase. However, in preparations cultured in the presence of serum, the number of peritubular cells was much greater in conventional Sertoli cell-enriched aggregates not treated with hyaluronidase (Table 1).

DISCUSSION

With immunocytochemical and immunochemical procedures, we have shown that fibronectin is produced by peritubular cells but not by purified preparations of Sertoli cells in culture. Antisera directed against fibronectin can be used to detect the presence of fibroblast-like cells in conventional Sertoli cell-enriched cultures, even when patches of peritubular cells are beneath the Sertoli cell monolayer, and therefore not visible by phase-contrast microscopy (Fig. 4B and D).

We have employed immunofluorescent techniques described to monitor the relative degree of contamination of various types of Sertoli cell-enriched preparations by peritubular cells. By subjecting conventional preparations to digestion with hyaluronidase, and by increasing the number of washes during the procedure, we have obtained more purified Sertoli cell-enriched aggregates (Table 1). These aggregates are relatively free of fibroblast-like cells, as judged by the absence of fibronectin deposition by Sertoli cells maintained in culture for 6 days in the presence of serum (Fig. 4A and C). In contrast, fibronectin deposition is clearly evident in conventional Sertoli cell-enriched preparations which have not been subjected to hyaluronidase (Figs. 4B,D and 5). In these preparations, peritubular cells present rapidly proliferate in the presence of serum (Table 1), unless inhibitors of DNA synthesis are added (Tung et al., 1980).

In conventional Sertoli cell preparations cultured in serum-free MEM, contaminating peritubular cells constitute approximately 5% when assessed by transmission electron microscopy (TEM) (Fritz et al., 1975), and 7.5% when analyzed by light microscopy of Feulgen-stained cells (Table 1 and Fig. 7). In the presence of serum, peritubular cells proliferate to constitute about 23% of the total present in conventional Sertoli cell-enriched aggregates in culture for 6 days. In contrast, in purified Sertoli cell-enriched preparations, the peritubular cells constitute less than 1% of the total cell population cultured for 6 days in the presence or absence of serum (Table 1). It therefore is apparent that hyaluronidase treatment of the Sertoli cell preparations significantly reduces the degree of contamination by peritubular cells, and that this becomes of greatest importance when cells are cultured in the presence of serum. It seems likely that proteins released into the medium by conventional Sertoli cell-enriched preparations maintained in culture in the presence of serum include products of both Sertoli cells and peritubular cells. Purified populations of Sertoli cells radiolabeled with [^{35}S] methionine do not secrete labeled fibronectin (Fig. 6).

On the other hand, peritubular cells radiolabeled with [^{35}S] methionine do secrete a considerable amount of labeled fibronectin, and also other proteins of high molecular mass (Fig. 6). Consequently, the appearance of fibronectin

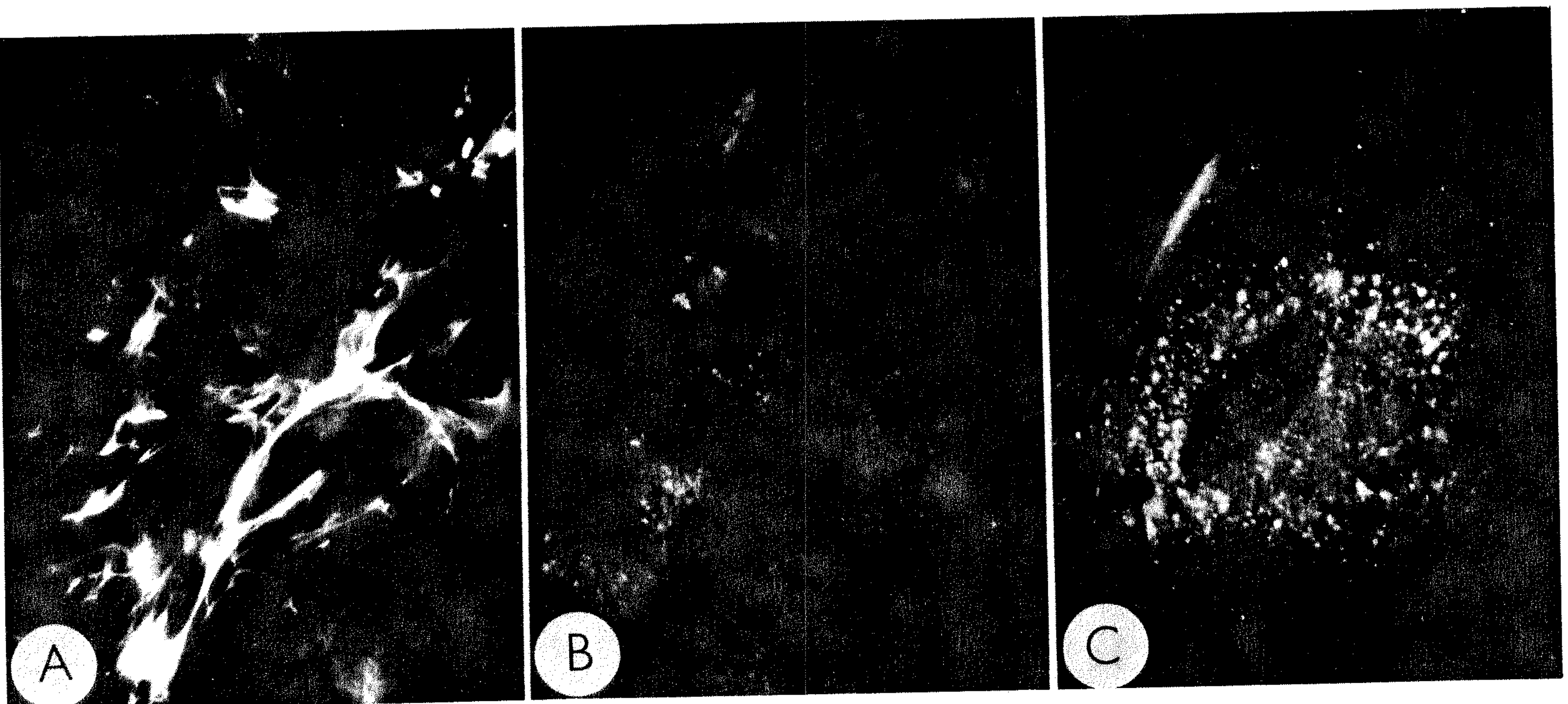
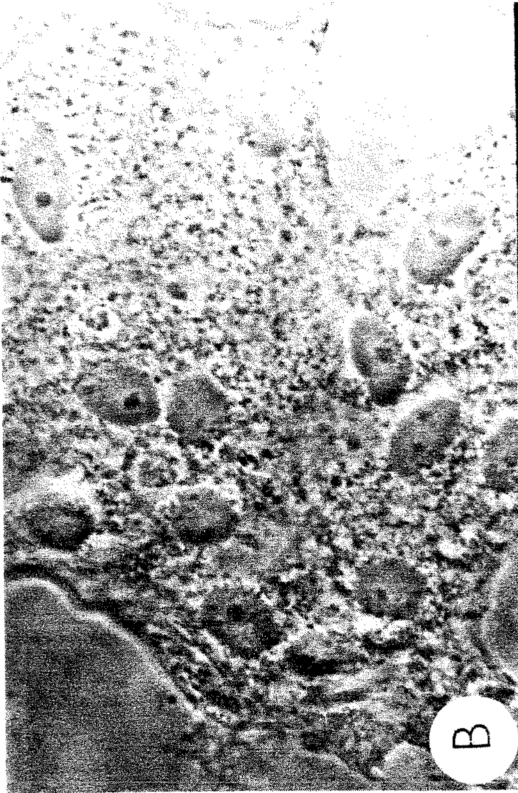


FIG. 3. Immunofluorescent micrographs of representative fields of secondary peritubular cell cultures. The culture had been maintained in modified MEM containing 10% calf serum (*A*), or MEM alone (*B*, *C*) for 4 days. Cells were then fixed and reacted with monoclonal fibronectin antiserum. Note deposition of filamentous fibronectin in the presence of serum (*A*). In the absence of serum, however, only intracellular components in the perinuclear region reacted with the antibody. A higher magnification of a field similar to that depicted in *B* is shown in *C*. Magnifications: A, B, ×1200; *C*, ×1900.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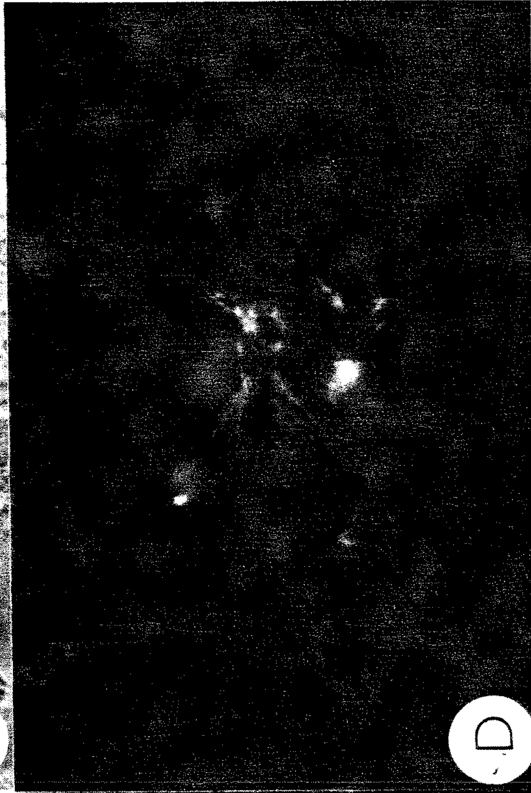
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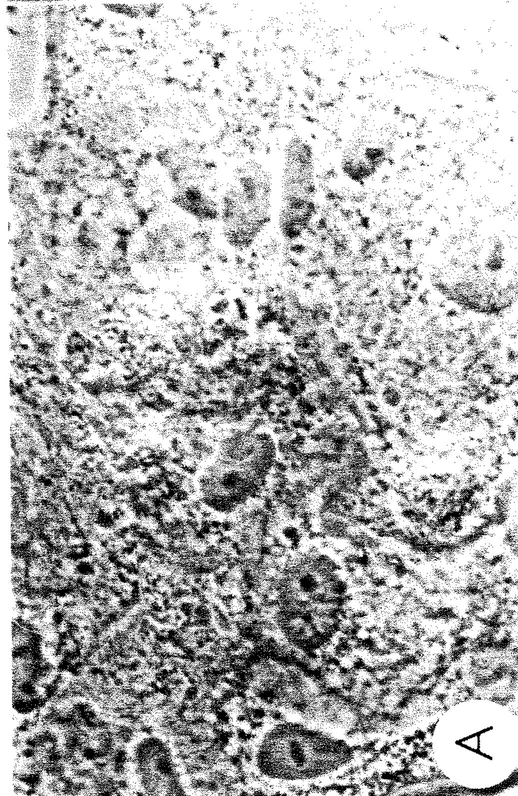
A

C

FIG. 4. Phase-contrast micrographs (*A*, *B*) and immunofluorescent micrographs (*C*, *D*) of confluent cultures of Sertoli cells. *A* and *C* show a representative field of a dense culture from a purified Sertoli cell-enriched preparation subjected to hyaluronidase treatment. *B* and *D* show a representative field of a confluent culture from a conventional Sertoli cell-enriched preparation which had not been digested with hyaluronidase. All cells had been cultured for 4 days in modified MEM containing 10% calf serum, fixed and reacted with monoclonal fibronectin antibody. ×1200.

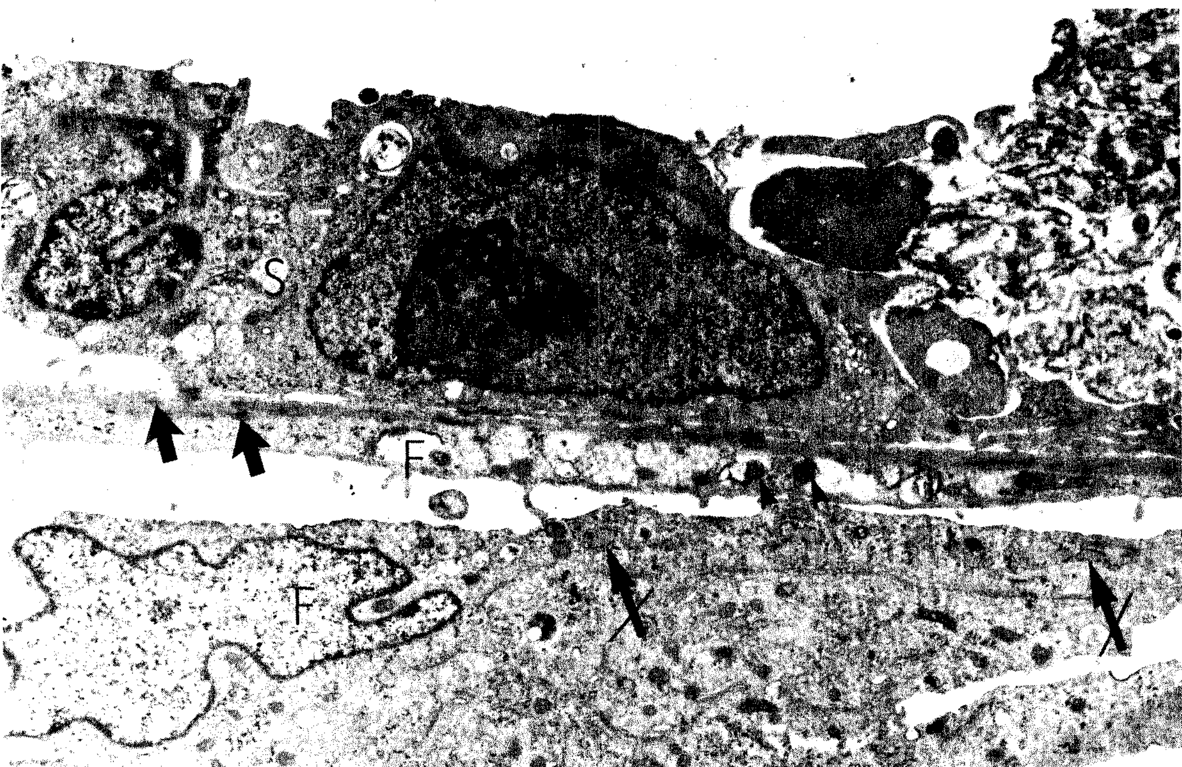

FIG. 5. Transmission electron micrograph of conventional Sertoli cell-enriched preparation not subjected to hyaluronidase treatment. Fibroblast-like peritubular cells (*F*) are evident beneath Sertoli cells (*S*). The cells had been cultured for 6 days in modified MEM containing 10% calf serum. This representative section, which is cut obliquely to the substratum, shows details of the endocytotic bodies (*arrowheads*), dilated cisternae (*cross arrows*) and dense bands of a microfibrillar network (*arrows*), all characteristic of peritubular myoid cells (Bressler and Ross, 1972, 1973). ×12500.

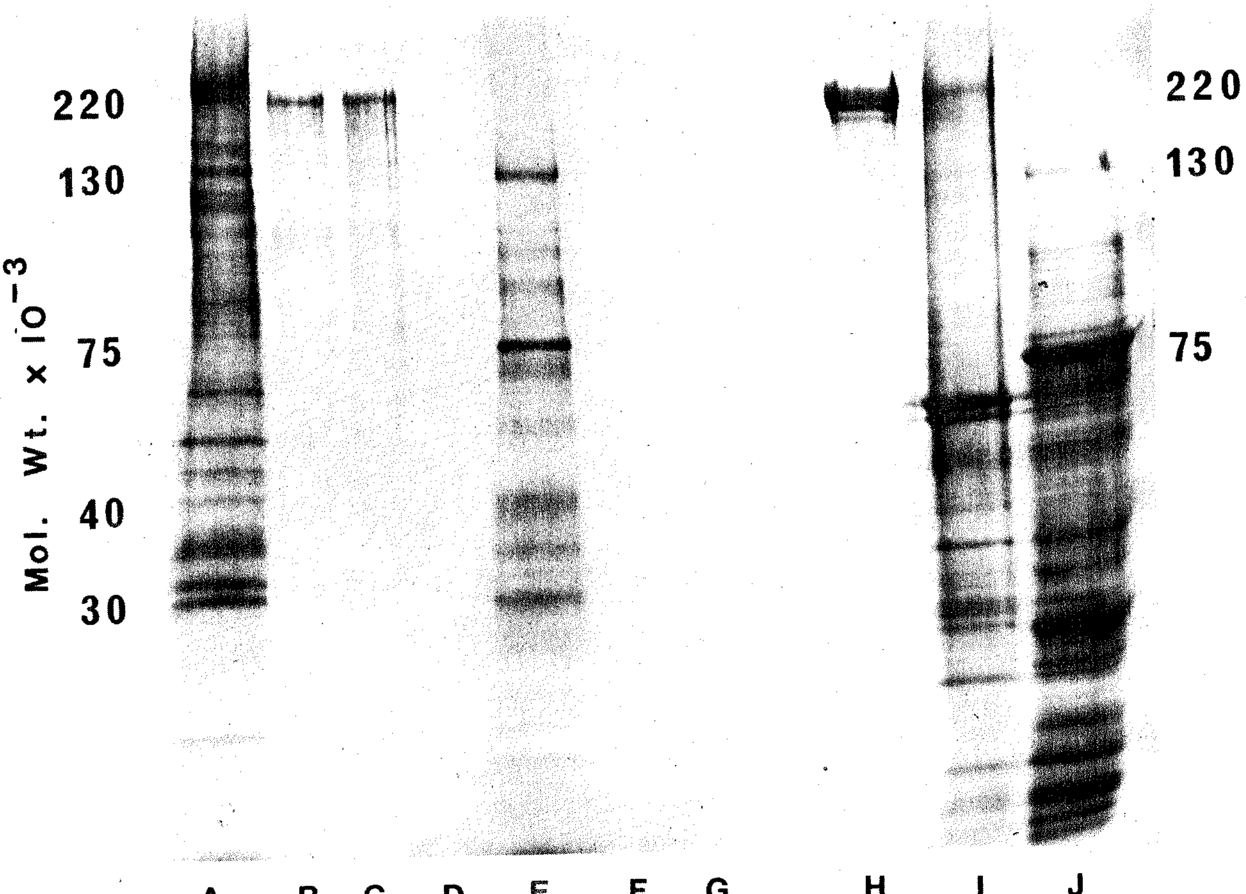

FIG. 6. Electrophoretic analysis of radiolabeled proteins synthesized by cultures of Sertoli or myoid cells, and immunoprecipitation by fibronectin antibody. An SDS 5 to 15% polyacrylamide gradient slab gel was either stained with Coomassie blue, (lanes *H* to *J*), or radiolabeled proteins were fluorographed (lanes *A* through *G*). The fluorograph lanes are: *A*) peritubular cell-secreted radiolabeled proteins; *B*) goat anti-rat fibronectin immunoprecipitate of peritubular cell secreted proteins; *C*) mouse anti-pig fibronectin immunoprecipitate; *D*) control nonimmune serum immunoprecipitates of peritubular cell secreted proteins; *E*) Sertoli cell-secreted proteins; *F*) goat anti-rat fibronectin immunoprecipitate of Sertoli cell secreted proteins; and *G*) control nonimmune serum immunoprecipitate of Sertoli cell-secreted proteins. The Coomassie blue-stained protein lanes are: *H*) rat fibronectin, *I*) peritubular cell secreted proteins; and *J*) Sertoli cell-secreted proteins.

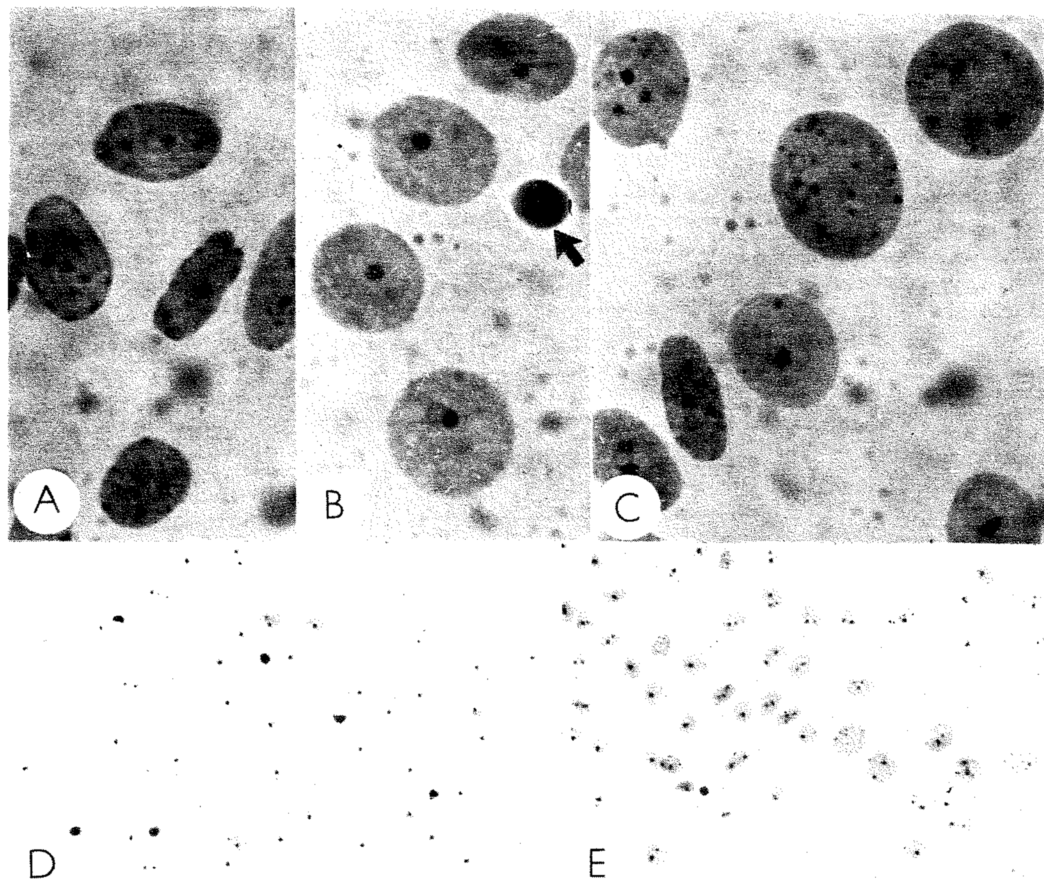

FIG. 7. Light micrographs of Feulgen-stained monolayers from a subculture of peritubular myoid cells (*A*), purified Sertoli cell-enriched aggregates subjected to hyaluronidase treatment during preparation (*B* and *D*), and a conventional Sertoli cell-enriched preparation (*C* and *E*). All cells had been maintained in modified MEM containing 10% calf serum for 4 days. *Arrow* indicates a germ cell remaining in the hyaluronidase-treated Sertoli preparation (*B*). Magnification: A, B, C, ×1900; D, E, ×600.

TABLE 1. Percentage of peritubular cells in Sertoli cell-enriched preparations cultured for 6 days in the presence and absence of serum.

Culture medium	Conventional Sertoli cell-enriched preparation	Hyaluronidase-treated Sertoli cell-enriched preparation
MEM	7.5% ± 0.88%[a]	0.3% ± 0.04%
MEM plus 10% calf serum	23.0% ± 1.73%	0.8% ± 0.06%

[a]Values are expressed as the mean ± SEM for 3000 cells counted in Feulgen-stained monolayers of three different Sertoli cell-enriched cultures in each class. For other details, see Fig. 7 and *Materials and Methods*.

with an M_r of approximately 220,000 in Sertoli cell-enriched preparations would indicate contamination by a significant number of peritubular cells. This is likely to occur in conventionally prepared Sertoli cell-enriched aggregates cultured in the presence of serum (Table 1 and Fig. 4B and D). In contrast, Sertoli cells radiolabeled with [^{35}S]methionine secrete ceruloplasmin having an M_r of 130,000 (Skinner and Griswold, 1983) and transferrin, having an M_r of 75,000 (Skinner and Griswold, 1980). Neither of these proteins is synthesized by peritubular cells. The SDS-polyacrylamide gel profiles of radiolabeled proteins, along with the cytochemical demonstration of fibronectin, thus provide convenient markers for the detection of peri-

tubular cells in Sertoli cell-enriched preparations. In this regard, it is of interest that a recent report by De Philip and Kierzenbaum (1982) on radiolabeled proteins synthesized by conventional Sertoli cell-enriched preparations, cultured for 6–8 days in the presence of 10% fetal bovine serum, indicates the presence of several proteins of molecular mass higher than 130,000 daltons including a band having an M_r of approximately 200 to 220 k. These high molecular weight proteins are likely to be products of contaminating peritubular cells, a possibility consonant with the pattern of proteins secreted by isolated populations of peritubular cells (Fig. 6, and see Fig. 3 of Kissinger et al., 1982).

Androgen addition to the medium stimulates the formation of androgen-binding protein (ABP) (Louis and Fritz, 1977; 1979), and transferrin (Skinner and Griswold, 1982) by Sertoli cell-enriched preparations. It has been demonstrated that peritubular cells in coculture with Sertoli cells stimulate Sertoli cells to produce more ABP (Tung and Fritz, 1980; Hutson and Stucco, 1981). Since the development of peritubular myoid cells in vivo is known to be dependent on androgens (Bressler and Ross, 1972), it is possible that peritubular cells present in conventional Sertoli cell-enriched preparations may respond to androgens in the medium, and possibly thereby alter Sertoli cell functions. With the availability of more homogeneous cell populations, it becomes possible to perform reconstitution experiments to establish whether androgen effects observed in Sertoli cell-enriched cultures are independent of androgen actions on peritubular cells.

Newly isolated peritubular cells did not react with fibronectin antibody, suggesting that the enzymatic treatment employed during cell preparations probably removed fibronectin from the cell surfaces. Fibronectin has been postulated to be required for attachment of many cells in culture to the substratum (Yamada and Olden, 1978). However, peritubular cells attached to the plastic surface well before fibronectin deposition became evident by immunofluorescent microscopy (Fig. 2B and E). While fibronectin in calf serum added to the medium may have been sufficient to permit cell attachment but insufficient to allow detection with fibronectin antiserum, results suggest that peritubular cells do not require fibronectin deposition from de novo fibronectin synthesis for cell attachment. Virtanen et al. (1982) have recently presented data indicating that fibronectin is not required for attachment of all cell types.

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